Hemorrhagic shock drives glycocalyx, barrier and organ dysfunction early after polytrauma

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Abstract: Polytrauma (PT) is frequently associated with hemorrhagic shock (HS), which increases morbidity and mortality. Although various aspects of HS have been addressed in PT patients, the impact of an additional HS is largely unknown regarding the development of multiple organ dysfunction associated with disturbed glycocalyx and barrier function early after trauma. A prospective, longitudinal, monocentered, observational study enrolling severely injured patients (Injury Severity Score, ISS=38.1±2.6) served for an in-depth analysis of blood (drawn on days 0, 1, 2, 3 and 5) and clinical data (up to 21days) of 30 patients who were then stratified into PT with and without HS. HS significantly enhanced signs of acute organ injury, assessed by increased serum concentrations of novel damage markers. Moreover, indicators of glycocalyx and tight-junction dysfunction were found in PT patients all of which were significantly enhanced in co-presence of HS. These markers revealed multiple significant correlations with specific barrier, fluid-balance, coagulation, inflammation, and clinical-outcome parameters. Strikingly, mucosa fragments, which affected clotting, could be detected in serum after PT/HS. The results point to HS as a main driver for glycocalyx and barrier breakdown and suggest novel tools for the monitoring of organ dysfunction in the early course after PT.

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Rebecca Halbgebauer [a], Christian K. Braun [a], Stephanie Denk [a], Benjamin Mayer [b], Paolo Cinelli [c], Peter Radermacher [d], Guido A. Wanner [c], Hans-Peter Simmen [c], Florian Gebhard [e], Daniel Rittirsch [c,1], Markus Huber-Lang [a,*,1]

[a] Institute of Clinical and Experimental Trauma-Immunology, University Hospital Ulm, Helmholtzstr. 8/1, 89081 Ulm, Germany
[b] Institute of Epidemiology and Medical Biometry, University of Ulm, Schwabstr. 13, 89075 Ulm, Germany
[c] Division of Trauma Surgery, Department of Surgery, Raemistr. 100, University Hospital Zurich, University of Zurich, 8091 Zurich, Switzerland
[d] Institute of Pathophysiology and Process Development in Anesthesia, University of Ulm, Helmholtzstr. 8/1, 89081 Ulm, Germany
[e] Department of Orthopedic Trauma, Hand-, Plastic- and Reconstructive Surgery, University Hospital Ulm, Albert-Einstein-Allee 23, 89081 Ulm, Germany

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ABSTRACT

Polytrauma (PT) is frequently associated with hemorrhagic shock (HS), which increases morbidity and mortality. Although various aspects of HS have been addressed in PT patients, the impact of an additional HS is largely unknown regarding the development of multiple organ dysfunction associated with disturbed glycocalyx and barrier function early after trauma.

A prospective, longitudinal, mono-centered, observational study enrolling severely injured patients (Injury Severity Score, ISS = 38.1 ± 2.6) served for an in-depth analysis of blood (drawn on days 0, 1, 2, 3 and 5) and clinical data (up to 21 days) of 30 patients who were then stratified into PT with and without HS.

HS significantly enhanced signs of acute organ injury, assessed by increased serum concentrations of novel damage markers. Moreover, indicators of glycocalyx and tight-junction dysfunction were found in PT patients all of which were significantly enhanced in co-presence of HS. These markers revealed multiple significant correlations with specific barrier, fluid-balance, coagulation, inflammation, and clinical-outcome parameters. Strikingly, mucosa fragments, which affected clotting, could be detected in serum after PT/HS.

The results point to HS as a main driver for glycocalyx and barrier breakdown and suggest novel tools for the monitoring of organ dysfunction in the early course after PT.

1. Introduction

Hemorrhagic shock (HS) is a frequent early event in severely injured polytrauma (PT) patients and, despite modern damage control and shock-treatment strategies, HS remains associated with high morbidity and mortality rates [1,2]. In PT patients and experimental PT settings, HS has been proposed as an engine of systemic inflammation [3], gut-barrier disruption [3,4], danger-associated molecule pattern (DAMP) exposure [5], endotheliopathy [6-8], complementopathy and coagulopathy [9-12]. All these mechanisms may contribute to the development of multiple organ dysfunction syndrome (MODS). Although various proteomic and transcriptomic data sets from PT patients have been analyzed for inflammatory and infectious consequences [13,14], mechanisms of the molecular danger response due to HS directing towards early MODS development remain rather unknown. Concerning therapeutic strategies for HS in trauma, novel concepts address not only the inflammatory axes [15] and coagulation network [16,17], but also focus in particular on the endotheliopathy, *e.g.* by application of fresh-

Abbreviations: AIS, Abbreviated Injury Scale; AKI, acute kidney injury; aPTT, activated partial thromboplastin time; CC16, Clara Cell Protein 16; DAMP, danger-associated molecule pattern; GCS, Glasgow Coma Scale; HS, hemorrhagic shock; I-FABP, liver −/intestinal-type fatty acid binding protein; ISS, Injury Severity Score; L-FABP, liver-type fatty acid binding protein; MMP-13, matrix metalloproteinase 13; MODS, multiple organ dysfunction syndrome; NGAL, neutrophil gelatinase-associated lipocalin; PCT, procalcitonin; PT, polytrauma; RBC, red blood cell; ROTEM, rotational thromboelastometry; S1P, sphingosine-1-phosphate; SOFA, Sequential Organ Failure Assessment; TASH, Trauma Associated Severe Hemorrhage.

* Corresponding author.
E-mail addresses: rebecca.halbgebauer@uniklinik-ulm.de (R. Halbgebauer), Christian.Braun@uni-ulm.de (C.K. Braun), Stephanie.Denk@uniklinik-ulm.de (S. Denk), Benjamin.Mayer@uni-ulm.de (B. Mayer), Paolo.Cinelli@usz.ch (P. Cinelli), Peter.Radermacher@uni-ulm.de (P. Radermacher), Guido.Wanner@sbk-vs.de (G.A. Wanner), HansPeter.Simmen@usz.ch (H.-P. Simmen), Florian.Gebhard@uniklinik-ulm.de (F. Gebhard), Daniel.Rittirsch@usz.ch (D. Rittirsch), Markus.Huber-Lang@uniklinik-ulm.de (M. Huber-Lang).
[1] Senior authors contributed equally.

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frozen plasma, adiponectin or prothrombin-complex concentrates to restore the vascular barrier [18,19]. Other treatment approaches aimed to improve trauma −/HS-induced intestinal hypoperfusion, mucosal ischemia and gut-barrier breakdown and thereby MODS development, for example, by the glycosaminoglycan heparan sulfate, which in addition to exhibiting some anti-coagulatory effects could improve immunological functions [20]. Nevertheless, a precautionary approach is required for novel pathophysiological and therapeutic concepts in trauma −/HS-induced MODS [21]. Consequently, the organ damage and barrier reactions after PT require closer discriminative characterization in regard to the presence or absence of an additional HS and using reliable monitoring markers.

2. Materials and methods

2.1. Clinical study design

A subcohort of 30 patients was randomly selected from a prospective, mono-centered, observational cohort study including a total of 104 patients. Blood was drawn consecutively during the first 21 days after PT when admitted to the University Hospital Zurich (Trauma Level I Center). The study was carried out in accordance with local and international guidelines and regulations and the study protocol was approved by the Cantonal Ethic Commission Zurich (StV 26–2007). All patients were recruited into the study under informed consent as approved by the Cantonal Ethic Commission Zurich and international ethical guidelines (NCT02508272). Enrollment criteria were ISS > 17, age ≥ 18 y, and time after injury <6 h. A clinico-transcriptomic approach for septic complications after PT as primary endpoint was recently published [13,22]. In the present study, the subcohort was stratified for manifest HS [23-25] on day 0 as indicated by at least one of the following parameters: base excess < −6 mmol/l, lactate ≥2.5 mmol/l, red blood cell (RBC) transfusion >2 U, and/or a Trauma Associated Severe Hemorrhage (TASH) score ≥ 10. The patient stratification strategy identified $n = 10$ patients without and $n = 20$ with signs of manifest HS. For baseline levels, six healthy age- and sex-matched volunteers were included after informed and written consent (mean age ± standard error of the mean (s.e.m.): 43.3 ± 8.3 y; median, min.–max.: 44.5, 18–72; male/female 4/2). All included study patients received standard treatment based on the guidelines of the German Society of Trauma (DGU).

2.2. Clinical data

Concurrent to the blood sampling, clinical data were collected prospectively. Corresponding scores were retrospectively determined from the patients' records. As recently described [13], various scores were applied: ISS, Glasgow Coma Scale (GCS) [26], Sequential Organ Failure Assessment (SOFA) score [27], systemic inflammation score [13], and the TASH score [23]. Systemic inflammatory response syndrome and sepsis were defined in accordance with previously established guidelines [28-30].

2.3. Enzyme-linked immunosorbent assay analyses of patient samples

Blood from patients and healthy volunteers was centrifuged at 2000×*g* for 10 min at 4 °C and serum was stored at −80 °C. Samples were assayed using the following kits for human proteins according to manufacturers' instructions: FABP2/I-FABP, Lipocalin-2/NGAL, Angiopoietin-2, Syndecan-1, C-reactive protein, MMP-13, serum albumin (all DuoSet ELISA, R&D, Wiesbaden-Nordenstadt, Germany), IL-6 (BD Biosciences, Heidelberg, Germany) CC16 (Clara Cell Protein 16, Elabscience, Bethesda, MD, USA), Heparan Sulfate (Amsbio, Abingdon, UK), L-FABP (Hycultec, Beutelsbach, Germany), Claudin-5 (Cusabio, College Park, MD, USA), ADM/Adrenomedullin (LifeSpan BioSciences, Seattle, WA, USA), MUC2 (Aviva Systems Biology, San Diego, CA, USA) and Sphingosine 1 Phosphate (MyBiosource, San Diego, CA, USA).

2.4. Rotational thromboelastometry (ROTEM) analyses

To assess the impact of dilution by high-volume resuscitation, whole blood anticoagulated with sodium citrate (Sarstedt, Nuembrecht, Germany) was drawn from healthy volunteers. The respective hematocrit was adjusted to 33% or 25% with Jonosterile (Fresenius Kabi, Bad Homburg, Germany) and incubated with heparan sulfate (Amsbio, Abingdon, UK), human recombinant syndecan-1 (R&D) and mucin-2 (Sigma-Aldrich, Darmstadt, Germany) for 30 min. The activity of the intrinsic pathway (INTEM test) was then analyzed using a ROTEM® delta device (Tem, Munich, Germany).

2.5. Statistics

Patient characteristics in the two groups were compared using the Chi-square test in case of categorical variables and using Student's *t*-test in case of continuous parameters. Correlation analyses were performed using Pearson Product Moment Correlation. ROTEM parameters of blood samples with adjusted hematocrit were compared using one-way ANOVA followed by Student-Newman-Keuls post-hoc test and paired t-test. These statistical analyses were performed using SigmaPlot (Version 11.0, Systat Software, Erkrath, Germany). To evaluate whether HS significantly changed overall plasma values of barrier and organ molecules during the time course after trauma, we used repeated-measures ANOVA employing SAS (Version 9.3, SAS, Cary, NC, USA). We applied no formal statistical test on normality due to their very limited validity regarding our available sample size [31]. Instead, we assessed graphically by means of QQ-plotting the model residuals whether there is suspicion of any severe violation of the model assumptions. Overall, we found no apprehensive deviation from the model assumptions for all the variables tested. The alpha level was 0.05 for all analyses. Results are presented as mean ± s.e.m.

3. Results

3.1. Patient cohort

When compared to the total cohort of 104 patients described by Rittirsch et al. [13], the 30 patients in our randomly selected subcohort were not significantly different regarding any demographic parameter (Table 1). Six age- and sex-matched volunteers were enrolled as healthy controls. We stratified the 30 patients into those with and those without clear signs of HS employing established clinical parameters [23-25]: base excess (< −6 mmol/l), lactate (≥2.5 mmol/l), RBC transfusion (>2 U on day 0), and/or TASH score (≥10) of which at least one had to be fulfilled to define HS. All key patient parameters are presented in Table 1. When comparing the two groups with ($n = 20$) and without ($n = 10$) HS, we found that although the ISS was similar in both groups (38.7 ± 2.8 *vs.* 36.2 ± 6.3, $p = 0.54$), the Abbreviated Injury Scale (AIS) of the abdomen/lumbar spine and the maximal SOFA until day 21 were significantly increased in the cohort with HS (2.8 ± 0.3 *vs.* 1.1 ± 0.4, $p = 0.01$, and 10.5 ± 1 *vs.* 6 ± 1.1, $p = 0.009$, respectively). Furthermore, the length of stay in the intensive care unit and in the hospital were significantly longer for patients with HS (19.2 ± 2.8 *vs.* 5.9 ± 1.3, p = 0.01, and 34 ± 4.1 *vs.* 17.2 ± 3.2, $p = 0.001$, respectively). As expected from our stratification, the data confirmed that HS patients had a higher initial TASH score ($p = 0.004$), lactate levels ($p = 0.007$) and hematocrit values ($p = 0.013$), required more total RBC transfusions ($p < 0.001$) and had impaired coagulation as indicated by reduced Quick values (calculated from the thromboplastin time; lower Quick relates to longer thromboplastin time) ($p = 0.008$) compared to patients without manifest HS (Table 1).

Table 1
Descriptive statistics and comparison of demographic parameters in total cohort and stratified groups. Statistical comparison of total *vs.* subcohort and of stratified groups respectively shown as *p*-values (Chi-square test for categorical variables and Student's *t*-test in case of continuous parameters).

	Polytrauma subcohort ($n = 30$)	Total cohort ($n = 104$) *vs.* subcohort	Polytrauma without HS (n = 10)	Polytrauma with HS (n = 20)	PT + HS *vs.* PT
Stratification of hemorrhagic shock			All criteria fulfilled: base excess ≥−6 mmol/l lactate <2.5 mmol/l pRBC ≤2 units on day 0 TASH score < 10 points	One or more criteria fulfilled: base excess <−6 mmol/l lactate ≥2.5 mmol/l pRBC >2 units on day 0 TASH score ≥ 10 points	
	Mean ± SEM; median (min. –max.)	*p*-value	Mean ± SEM; median (min. –max.)	Mean ± SEM; median (min. –max.)	*p*-value
Demographics					
Age [y]	39.9 ± 3.3; 33.5 (18–80)	0.34	44.4 ± 6.3; 44.5 (21–78)	37.6 ± 3.8; 31.5 (18–80)	0.45
Sex [male/total]	21/30 (70%)	0.65	4/10 (40%)	17/20 (85%)	**0.011**
GCS	11.8 ± 0.8; 13.0 (3–15)	0.9	11.5 ± 1.5; 14 (3–15)	12 ± 0.9; 14 (3–15)	0.93
Traumatic brain injury (GCS ≤ 12)	11/30 (36.7%)	0.83	4/10 (40%)	7/20 (35%)	0.789
AIS max.	4.5 ± 0.2; 5 (2–6)	0.06	4.6 ± 0.9; 5 (3–6)	4.5 ± 0.8; 5 (3–6)	0.7
AIS head/neck/cervical spine	2.4 ± 0.4; 2 (0–6)	0.64	2.9 ± 0.9; 3 (0–6)	2.25 ± 0.4; 2 (0–6)	0.45
AIS face	0.6 ± 0.2; 0 (0–3)	0.64	0.3 ± 0.3; 0 (0–2)	0.8 ± 0.2; 0 (0–3)	0.2
AIS thorax/thoracic spine	3.6 ± 0.3; 4 (0–5)	0.09	3.3 ± 0.6; 3.5 (0–5)	3.7 ± 0.3; 4 (0–5)	0.49
AIS abdomen/lumbar spine	2.3 ± 0.3; 2 (0–5)	0.33	1.1 ± 0.4; 1 (0–3)	2.8 ± 0.3; 2.5 (0–5)	**0.01**
AIS upper/lower extremity	2.6 ± 0.2; 3 (0–5)	0.35	2.3 ± 0.4; 2.5 (0–3)	2.8 ± 0.2; 3 (1–5)	0.23
ISS	38.1 ± 2.6; 36 (17–75)	0.06	36.2 ± 6.3; 35 (17–75)	38.7 ± 2.8; 36 (19–75)	0.54
SOFA score initial	5.3 ± 0.6; 6 (0−11)	0.44	4.6 ± 1.2; 5 (0−10)	5.7 ± 0.7; 6.5 (0–11)	0.4
SOFA score max.	9 ± 0.8; 9 (1–18)	0.13	6 ± 1.1; 6.5 (1−11)	10.5 ± 1; 11 (1–18)	**0.009**
Outcomes					
RISC [% survival]	79.7 ± 4.9; 94.2 (25.5–98.8)	0.50	79.1 ± 10.2; 97.3 (25.9–98.8)	80 ± 5.7; 89.8 (25.5–98.5)	0.52
Survival	77% (7 non-survivors)	0.15	3/10 (30%)	4/20 (20%)	0.542
Hospital length of stay [d]	28.4 ± 3.2; 24.5 (4–82)	0.63	17.2 ± 3.2; 17.5 (4–40)	34 ± 4.1; 36.5 (4–82)	**0.01**
Intensive care unit length of stay [d]	15.1 ± 2.24; 12 (2–43)	0.68	5.9 ± 1.3; 4 (2−12)	19.2 ± 2.8; 16 (3–43)	**0.001**
Allogenic blood transfusions					
TASH score [points]	8.6 ± 1; 8 (0−22)	0.94	4.6 ± 0.9; 5 (0–8)	10.5 ± 1.2; 11 (2−22)	**0.004**
Initial (d0) pRBC transfusion [units]	5.6 ± 1.2; 2.5 (0−21)	0.33	1.1 ± 0.4; 1 (0–3)	7.9 ± 1.5; 5.5 (0–21)	**0.002**
Total pRBC transfusion [units]	13.4 ± 2.7; 7.5 (0–66)	0.18	3.8 ± 0.5; 4.5 (0–5)	18.2 ± 3.5; 15.5 (3–66)	<**0.001**
Massive transfusion rate	6/30 (20%)	0.39	0/10	6/20 (30%)	0.053
Infectious complications					
Nosocomial infections	19/30 (63.3%)	0.41	4/10 (40%)	15/20 (75%)	0.872
Sepsis	6/30 (20%)	0.57	0/10	6/20 (20%)	0.053
Hemostasis and blood gas analysis					
Initial base excess [mmol/l]	−3.1 ± 0.5; −2.4 (−9.8–2.0)	0.91	−2.2 ± 0.4; −2.4 (−3.7 to −0.1)	−3.6 ± 0.7; −3 (−9.8–2)	0.38
Initial lactate [mmol/l]	2.3 ± 0.3; 2.2 (0.7–6.8)	0.95	1.5 ± 0.2; 1.4 (0.7–2.3)	2.7 ± 0.3; 2.5 (1–6.8)	**0.007**
Initial Quick [%]	60.1 ± 3.7; 59 (22–98)	0.85	73.5 ± 5.7; 70 (48–98)	53.4 ± 4.1; 52.5 (22–95)	**0.008**
Initial aPTT [s]	40.6 ± 4.5; 33 (22–141)	0.87	29.7 ± 1.5; 29 (22–36)	46.1 ± 6.4; 36.5 (25–141)	**0.04**
Initial hematocrit [%]	28.9 ± 1.3; 29.8 (10.7–41.9)	0.86	33.5 ± 1.5; 33.7 (24.7–40.9)	26.6 ± 1.7; 26.7 (10.7–41.9)	**0.013**
Initial temperature [°C]	35.3 ± 0.2; 35 (33.1–38.8)	0.97	35.1 ± 0.2; 34.8 (34.2–36.3)	35.4 ± 0.3; 35 (33.1–38.8)	0.58

P-values <0.05 are bold.
Abbreviations: AIS, Abbreviated Injury Score; aPTT, partial thromboplastin time; GCS, Glasgow Coma Scale; HS, hemorrhagic shock; ISS, Injury Severity Score; pRBC, perfused red blood cells; PT, polytrauma; RISC, Revised Injury Severity Classification; SOFA score, Sequential Organ Failure Assessment score; TASH score, trauma associated severe hemorrhage score.

3.2. Markers of organ damage and correlation with clinical parameters

To estimate the effect of HS on organs, we determined the plasma levels of several markers that are considered to reliably represent organ function and integrity. Over the course of five days, HS significantly increased CC16 plasma levels as a proposed marker for lung damage compared to patients without HS ($p = 0.0498$, Fig. 1a). Although CC16 levels did not correlate with the AIS thorax (Supplementary Table S1), initial plasma levels were correlated with total RBC units ($r = 0.62$, $p = 0.001$, Fig. 1b) and plasma CC16 on day 1 was associated with the fluid balance on day 2 ($r = 0.65$, $p < 0.001$, Fig. 1c). Plasma neutrophil gelatinase-associated lipocalin (NGAL) as a marker for kidney injury was initially similar in both groups, but rose at later time points and was overall significantly higher in HS patients ($p = 0.0003$, Fig. 1d). Furthermore, higher NGAL levels on day 1 were significantly correlated with the AIS abdomen ($r = 0.66$, $p < 0.001$, Fig. 1e) and were associated with increased procalcitonin (PCT) levels on day 3 ($r = 0.85$, $p < 0.001$, Fig. 1f). Intestinal-type fatty acid binding protein (I-FABP) as a marker for intestinal damage and renal dysfunction was initially increased in HS patients and, although dropping afterwards, remained higher than in patients without HS throughout the five days ($p = 0.019$, Fig. 1g). Plasma I-FABP on day 0 positively correlated with lactate levels ($r = 0.71$, $p < 0.001$, Fig. 1h) and the fluid balance ($r = 0.51$, $p = 0.008$, Fig. 1j). To evaluate liver damage, we determined plasma liver-type FABP (L-FABP) and found significantly increased levels throughout the time course in the HS group ($p = 0.004$), whereas the amount in the

group without HS returned to the control value after day 1 (Fig. 1k). Higher L-FABP on day 1 correlated with lower Quick values ($r = -0.45$, $p = 0.013$, Fig. 1l), but with increased PCT on day 3 ($r = 0.80$, $p < 0.001$, Fig. 1m). Further correlations of organ-damage markers and clinical parameters until day 3 are listed in Supplementary Table S1.

3.3. Detection of systemic inflammation and dilution markers and endothelial barrier and glycocalyx components

In order to evaluate systemic inflammation, we detected IL-6 in the time course after injury and found significantly increased concentrations in the patient cohort with HS ($p = 0.001$, Fig. 2a). As a result of massive transfusion, serum albumin was decreased, although not significantly, after HS in comparison to patients without shock or healthy controls (Fig. 2b). To investigate whether HS exacerbated the traumatic damage to endothelial integrity with respect to loosening cell-cell contacts and disruption of the glycocalyx layer, we quantified several components in serum samples. Sphingosine-1 phosphate (S1P), a central regulator of vascular permeability, was decreased in all patients compared to healthy controls and there was a small difference between the trauma groups ($p = 0.024$, Fig. 2c). Matrix metalloproteinase 13 (MMP-13) was significantly higher in serum from HS

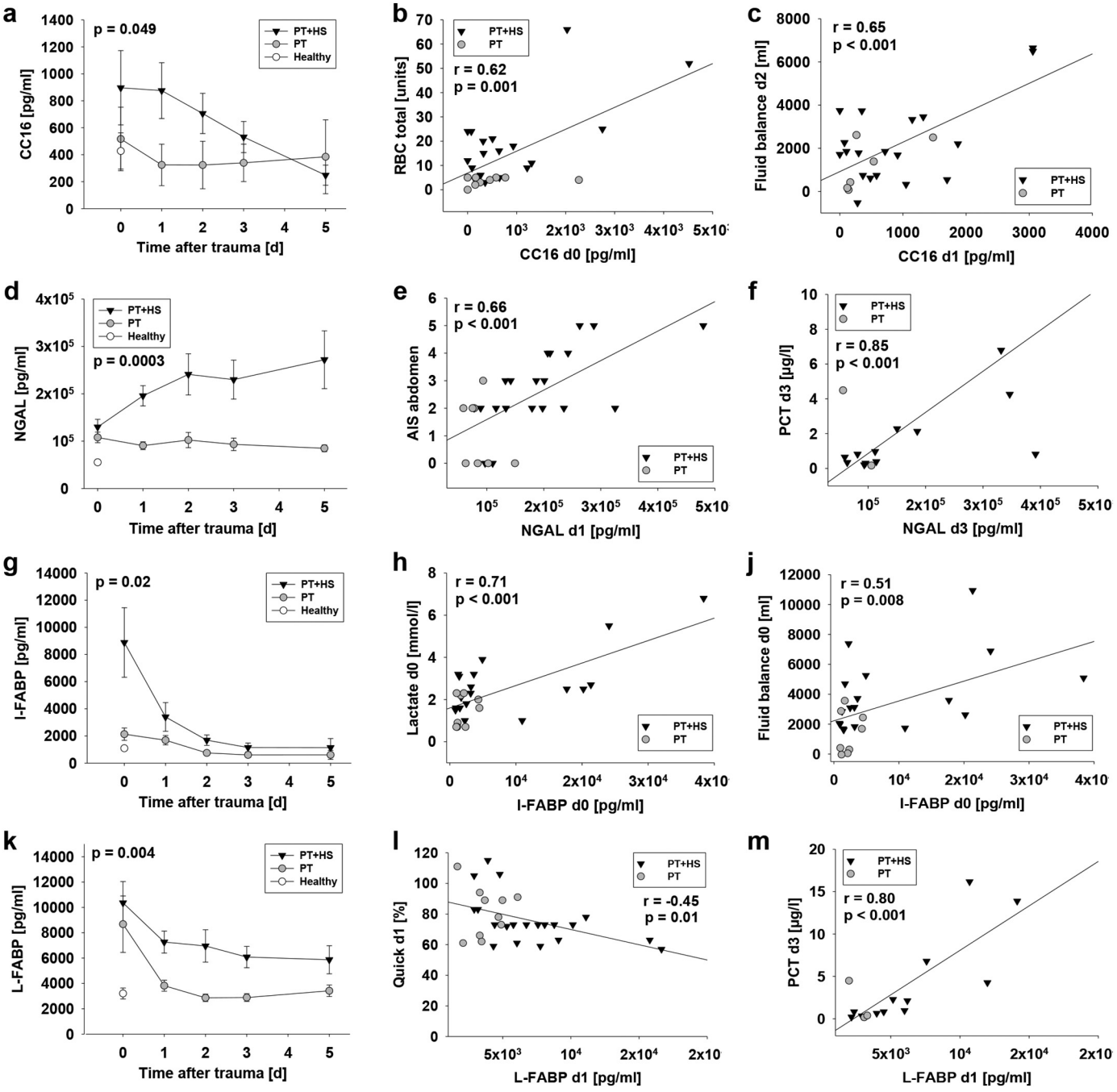

Fig. 1. Organ damage markers in the plasma of polytrauma patients and correlation with clinical parameters. Levels of the organ-damage markers a, clara cell secretory protein (CC16), d, neutrophil gelatinase-associated lipocalin (NGAL), g, intestinal fatty acid-binding protein (I-FABP) and k, liver-type fatty acid-binding protein (L-FABP) in plasma of healthy volunteers and polytrauma patients without and with HS. Repeated-measures ANOVA; $p < 0.05$ indicates significantly altered overall plasma values in the HS group compared to polytrauma alone. b, c, e, f, h, j, l, m, Pearson Product Moment Correlation of plasma levels of damage markers to clinical parameters in polytrauma patients without and with HS. Results are shown as mean ± s.e.m. AIS, abbreviated injury score; RBC, red blood cells; PCT, procalcitonin; r, Pearson correlation coefficient.

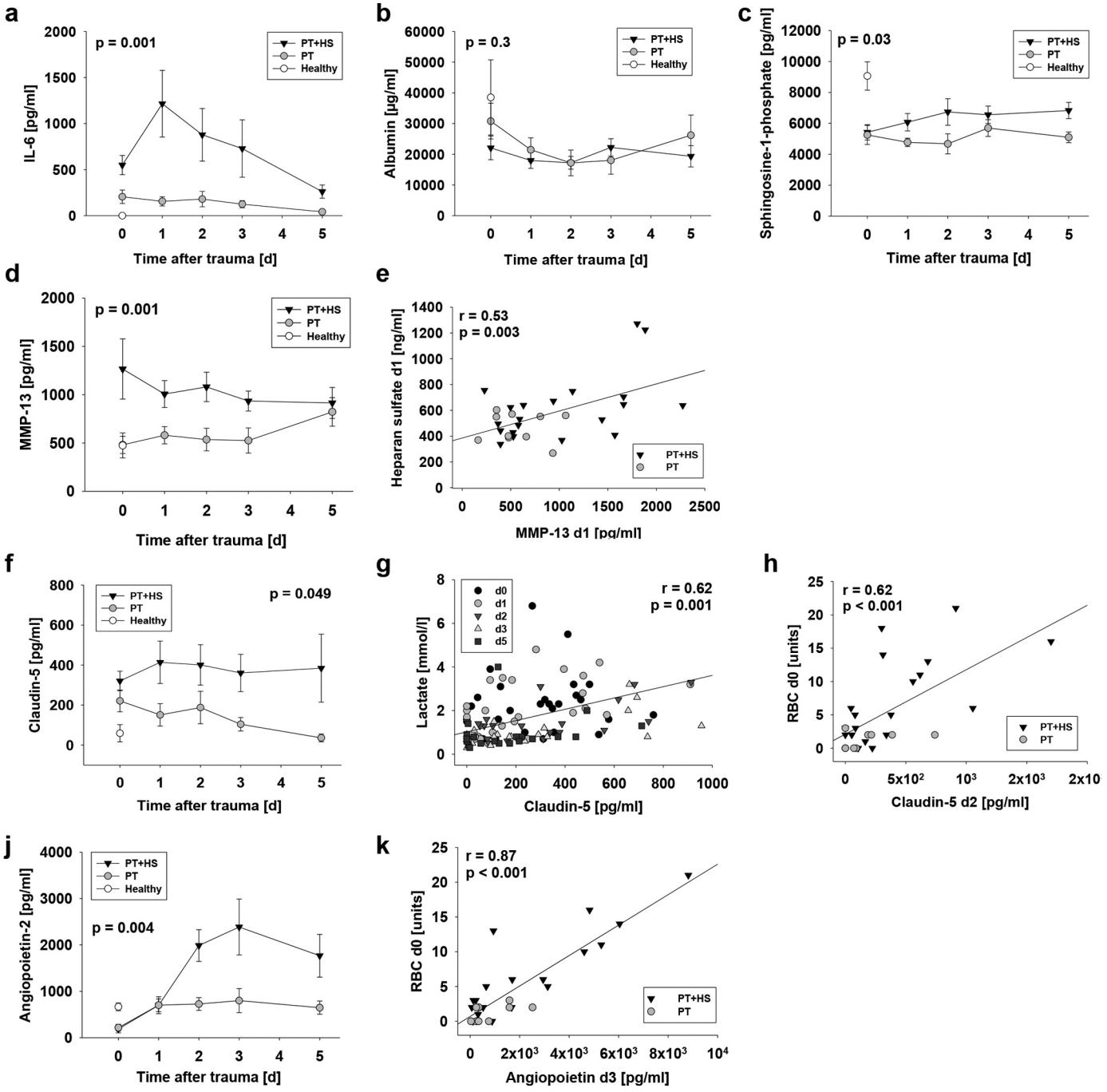

Fig. 2. Inflammatory markers and regulators of the vascular barrier in patient serum and correlation with clinical parameters. Concentrations of a, interleukin (IL)-6, b, albumin, c, sphingosine-1-phosphate, d, matrix metalloproteinase-13 (MMP-13), f, claudin-5, and j, angiopoietin-2 in serum of healthy volunteers and polytrauma patients without and with HS. Repeated-measures ANOVA; $p < 0.05$ indicates significantly altered overall serum concentrations in the HS group compared to polytrauma alone. g, Pearson Product Moment Correlation of plasma claudin-5 to lactate levels on days 0, 1, 2, 3 and 5. h, serum claudin-5 concentrations of polytrauma patients without and with HS on day 2 correlate with red blood cell (RBC) units on day 0. k, Pearson Product Moment Correlation of plasma angiopoietin-2 levels on day 3 with RBC on day 0. Results are shown as mean ± s.e.m. R, Pearson correlation coefficient.

patients ($p = 0.001$, Fig. 2d) and correlated with heparan sulfate concentrations on day 1 ($r = 0.53$, $p = 0.003$, Fig. 2e). Claudin-5, part of endothelial tight junctions that play an essential role in the blood-brain barrier function, was found at significantly increased levels in the plasma of HS patients ($p = 0.002$) and remained elevated throughout the 5-day observation period (Fig. 2f). Claudin-5 plasma levels positively correlated with blood lactate levels ($r = 0.63$, $p < 0.001$, Fig. 2g), and high numbers of transfused RBC units on day 0 were associated with higher claudin-5 levels even 2 days after injury ($r = 0.63$, $p < 0.001$, Fig. 2h). Angiopoietin-2, which is released by activated endothelium and sensitizes the endothelial cell in an autocrine fashion for inflammatory stimuli, was initially at the same level in both groups, but significantly increased from day 2 after HS and was overall higher in the HS group ($p = 0.004$, Fig. 2j). Interestingly, the extent of initial transfusion of patients (RBC on day 0) appeared to increase angiopoietin-2 even 3 days after trauma, as can be seen by the strong positive correlation ($r = 0.87$, $p < 0.001$, Fig. 2k).

Syndecan-1 as a component of the intravascular endothelial glycocalyx with manifold interactions with coagulatory processes was strongly increased in patients with HS over the time course of observation (p

< 0.0001, Fig. 3a), which was also the case when normalized to plasma protein ($p < 0.0001$, Fig. 3b). Higher syndecan-1 levels on day 1 were associated with higher maximal SOFA scores ($r = 0.56$, $p = 0.001$, Fig. 3c). Furthermore, plasma syndecan-1 levels over the course of the observation correlated significantly with the activated partial thromboplastin time (aPTT) ($r = 0.62$, $p < 0.001$, Fig. 3d) and weakly with plasma NGAL ($r = 0.40$, $p < 0.001$, Fig. 3e) and plasma L-FABP ($r = 0.39$, $p < 0.001$, Fig. 3f). When assessing heparan sulfate, we found only a slight increase in plasma levels of HS patients compared to those without HS (Fig. 3g), which was relativized by normalizing to plasma protein ($p = 0.003$, Fig. 3h). However, in *in vitro* experiments performing ROTEM analyses of whole blood with normal hematocrit (45%), addition of heparan sulfate dose-dependently altered coagulation parameters, especially the clotting time ($p < 0.05$, Fig. 3j). As expected, *in vitro* simulation

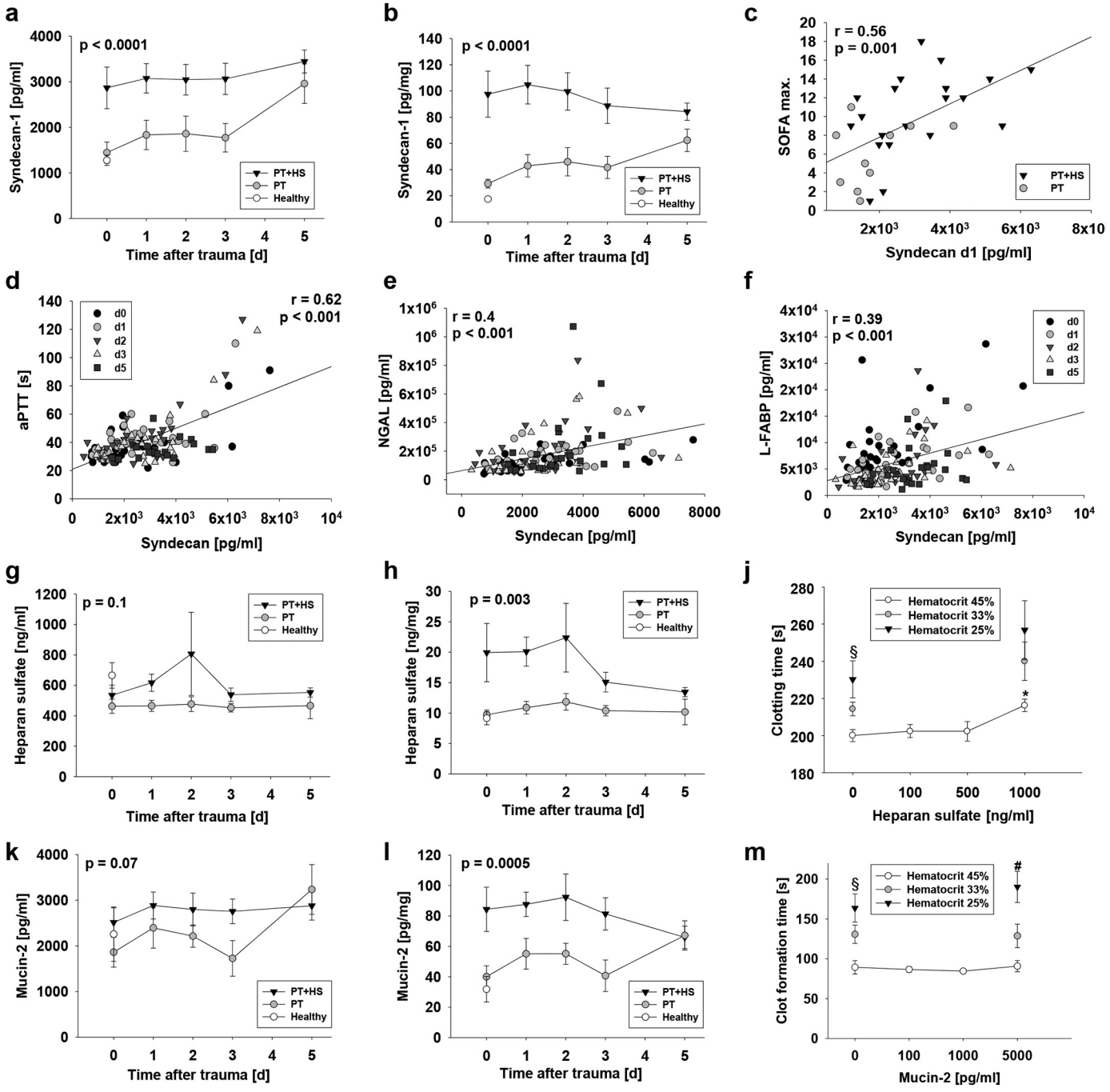

Fig. 3. Markers of barrier breakdown in the plasma of polytraumatized patients in correlation with clinical parameters and organ damage markers and their interference with coagulation. Plasma levels of a, syndecan-1, g, heparan sulfate and k, mucin-2 in plasma of healthy volunteers and polytrauma patients without and with HS. Repeated-measures ANOVA; $p < 0.05$ indicates significantly altered overall plasma values in the HS group compared to polytrauma alone. Plasma concentrations of b, syndecan-1, h, heparan sulfate and l, mucin-2 normalized to plasma protein. Repeated-measures ANOVA; $p < 0.05$ indicates significantly altered overall values in the HS group compared to polytrauma alone. c, Pearson Product Moment Correlation of plasma syndecan-1 on day 1 with the maximal sequential organ failure assessment score (SOFA max.) of polytrauma patients without and with HS. Pearson Product Moment Correlation of plasma syndecan-1 with d, the abbreviated prothrombin time (aPTT), e, plasma neutrophil gelatinase-associated lipocalin (NGAL) concentrations and f, plasma liver-type fatty acid-binding protein (L-FABP) levels of all patients on days 0, 1, 2, 3 and 5. ROTEM clot formation time of citrated blood from healthy volunteers with normal (45%) or adjusted (33% and 25%) hematocrit after incubation with j, heparan sulfate and m, mucin-2; *, $p < 0.05$ *vs.* hematocrit 45% without heparan sulfate, one-way ANOVA; §, $p < 0.05$ *vs.* hematocrit 45% without heparan sulfate/mucin-2, one-way ANOVA; #, $p = 0.005$ *vs.* hematocrit 25% without mucin-2, paired *t*-test. Results are shown as mean ± s.e.m. R, Pearson correlation coefficient.

of the different hematocrit values determined in our PT cohorts with and without HS (25% and 33%, respectively) significantly prolonged clotting time and clot formation time ($p < 0.05$, Fig. 3j and m) which was more apparent, although not significant, in the presence of 1000 ng/ml heparan sulfate (Fig. 3j). Furthermore, mucin-2 derived from the intestinal mucosa was slightly higher in the plasma of patients with HS assessed per ml serum ($p = 0.067$, Fig. 3k) and significantly enhanced when normalized to serum protein ($p = 0.0005$, Fig. 3l). *In vitro*, mucin-2 did not change clotting parameters in amounts detected in patient sera at a normal hematocrit, but was able to significantly prolong clot formation time when added to blood with an hematocrit of 25% ($p = 0.005$, Fig. 3m). Our main findings are summarized in Fig. 4.

4. Discussion

The pathophysiological and molecular danger response following PT appears aggravated in the presence of HS and remains mechanistically enigmatic and still rather challenging to treat. Recently, novel markers have been introduced to evaluate hidden early organ damage and for rapid detection and reliable monitoring of secondary organ damage as well as inflammatory/infectious and coagulopathic complications. CC16, NGAL, L-FABP and I-FABP were proposed to estimate the extent of lung contusion [32], kidney injury [33] and liver and intestinal damage [34], respectively. However, to date it remains unclear to what extent these proposed organ-specific markers are influenced by an additional HS in the complex setting of PT.

In contrast to reported correlations of CC16 with the volume of contused lung parenchyma in PT patients [32], the specificity of CC16 as a marker of lung damage is not supported by the present data. Other studies showed that higher CC16 may reflect a loss of air-blood barrier integrity [35], which is likely to happen in HS patients.

The central role of the kidneys in shock is evidenced by a study in >2000 trauma patients of whom >2% developed evident acute kidney injury (AKI) early after trauma, associated with MODS development in >75% of patients with AKI and a poor outcome [36]. This is in line with the early increase in NGAL serum concentrations in our HS cohort. Since HS-induced hypoperfusion and hypoxia contribute to tubular injury [37], it can be assumed that HS after PT causes early remote AKI *via* shock pathomechanisms on a prerenal level. Of note, NGAL values correlated with AIS abdomen during the early posttraumatic course even though a primary hit of the kidneys by the inflicted trauma force vector is rare. Therefore, PT-induced remote AKI as a "harbinger of poor outcome" [36] appears to be induced or aggravated by HS. Furthermore, association of NGAL values with PCT three days after PT may be due to infectious complications, including septic AKI known to be triggered by both pathogen-associated molecular patterns and DAMPs [38].

In regard to abdominal injury markers, L-FABP as a liver-damage marker [34,39] was increased in manifest HS and, as expected, correlated with coagulation function and indicators of acute phase infection. Intestinal barrier dysfunction assessed by serum I-FABP [34] exhibited a HS-dependent rapid and robust increase in PT patients which correlated with serum lactate and the fluid balance. It is striking that a recent prospective study enrolling severely burned patients stratified for MODS *vs.* no-MODS development showed an almost superimposable I-FABP course early after burn injury [40]. The data are suggestive of an HS-driven barrier and organ failure based on intestinal ischemia.

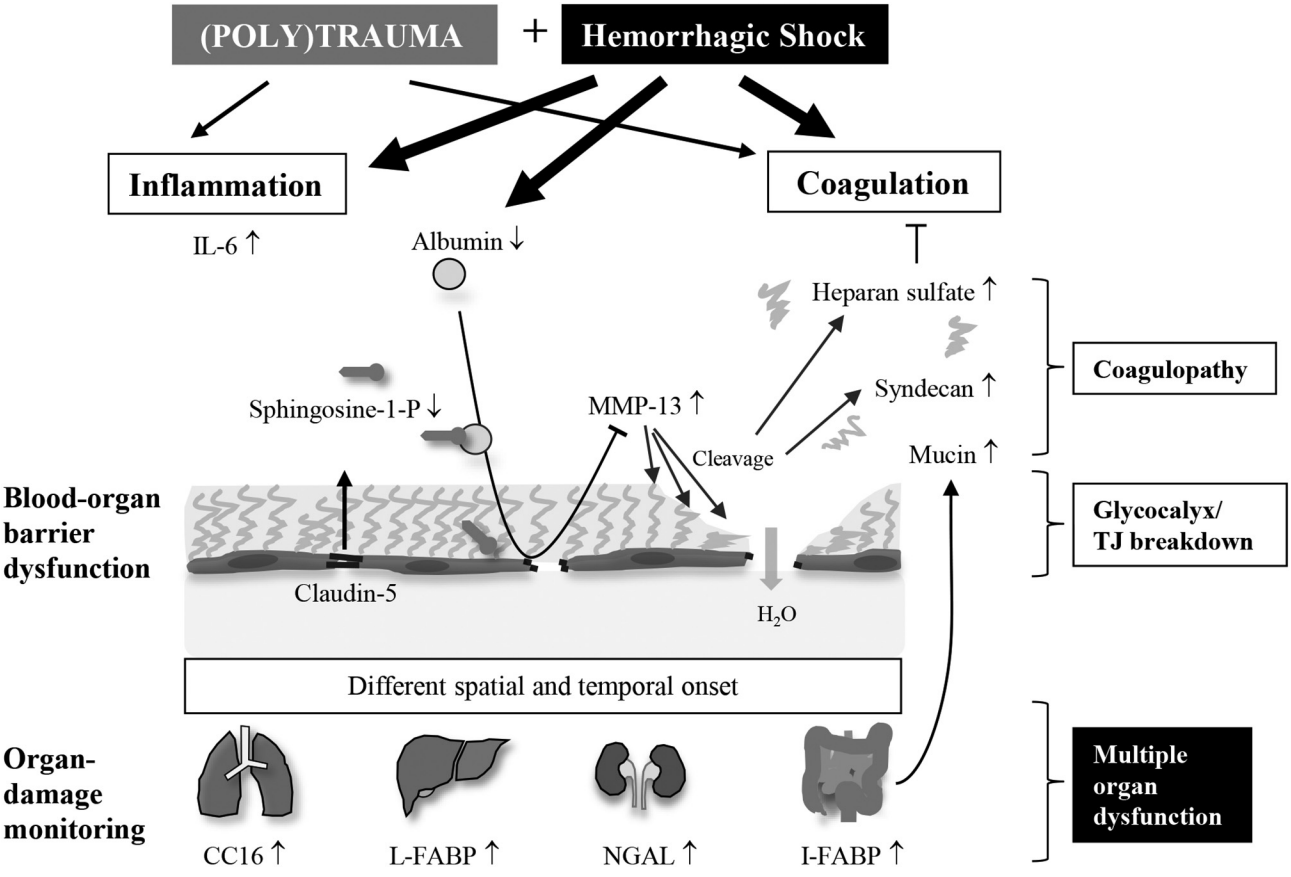

Fig. 4. Proposed role of hemorrhagic shock after clinical polytrauma. Hemorrhagic shock after polytrauma reinforces the inflammatory reaction. Massive transfusion due to blood loss leads to decreased albumin and sphingosin-1-phosphate concentrations, inducing an increase in matrix metalloproteinase 13 (MMP-13) by endothelial cells. MMP-13 can cleave off components of the endothelial glycocalyx which may interfere with coagulation. Furthermore, intestinal damage leads to the release of mucin into systemic blood with anticoagulatory features. The breakdown of glycocalyx and endothelial tight junctions (TJ, monitored by serum claudin-5) leads to organ damage in distinct spatial and temporal patterns which can be monitored by organ-specific clinical markers. CC16, Clara cell protein 16; I-FABP, intestinal fatty acid-binding protein; IL-6, interleukin-6; L-FABP, liver-type fatty acid-binding protein; NGAL, neutrophil gelatinase-associated lipocalin; sphingosine-1-P, sphingosine-1-phosphate.

Of note, after the traumatic hit, the different organs appear to follow a different time pattern for further organ-injury development as proposed recently [41]. A major driver for (multiple) organ dysfunction is the breakdown of blood-organ barriers. Recently, we published first evidence of circulating tight-junction molecules in humans and mice early after PT [42]. Therefore, plasma claudin-5 was used as a tight-junction marker and was detected persistently in the PT patients with manifest HS in contrast to decreasing concentrations in the absence of HS. The positive correlation between claudin-5 and initial lactate values and RBC transfusion units may indicate ischemia-induced breakdown of tight-junction barriers after trauma [4,43].

The endothelium also plays a central role in the pathophysiology after traumatic HS. In this context, the lipid mediator S1P is not only important for various immune functions, but in addition critical for vascular barrier integrity and coagulation [44], and has already been successfully applied in trauma/HS [20]. Angiopoietin-2 was introduced as promotor for vascular leakage and inflammation and was proposed to be predictive for septic complications and poor outcome [45]. To what extent angiopoietin-2 is involved in neoangiogenesis and regeneration of damaged tissues after PT/HS is relatively unknown and needs further investigation. Increased vascular permeability during the time course following PT and HS has been reported to involve degradation of the apical glycocalyx of endothelial cells [46], including syndecan-1 as an established biomarker for glycocalyx disruption [7,8,47] and its fragment heparan sulfate. Mechanistically, a prospective observational study of >400 severely injured patients (median ISS = 17) suggested sympathoadrenal activation as an important trigger for the development of endotheliopathic consequences, identifying epinephrine as the only independent predictor for syndecan-1 concentrations generated from glycocalyx shedding [6]. Because endothelial damage was strongly associated with thrombelastographic signs of coagulopathy in a recent prospective sepsis study [47], we assessed the thrombelastographic features in the presence of heparan sulfate in concentrations found in the blood of our PT cohort and under simulated conditions of hemorrhage- and resuscitation-induced hemodilution (hematocrit 25%) in comparison to PT without HS (hematocrit 33%) or healthy conditions (hematocrit 45%). Therefore, the described PT-induced "autoheparinization" effects [7] of shed glycocalyx structures, including heparan sulfate, could be worsened in the presence of HS. It seems that PT/HS-induced MMP-13 release induced by decreased albumin/S1P concentrations is responsible for the cleavage of glycocalyx components off the endothelial cells [48]. Aside from that, following HS in rodents, pancreatic enzymes appear to disrupt intestinal mucus layers and thereby play a detrimental role in gut-derived sepsis [49]. Here, we indeed detected mucin-2 in the blood of PT patients when HS was evident (up to 6000 pg/ml). Furthermore, mucin-2 appears to prolong clot-formation time in the setting of hemodilution and may thereby support coagulopathic development after trauma with HS.

In conclusion, the results suggest HS as a forceful driver for glycocalyx, barrier, and organ dysfunction in the early course after PT. In the future, early monitoring and detection of barrier failure is needed to allow for treatment of evident and occult organ damage before first manifestation of leakage syndrome and multiple-organ failure.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcrc.2017.11.025.

Conflicts of interest

None.

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